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Chapter 5

Signal Transduction in T Cell Activation and Tolerance

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SIGNAL TRANSDUCTION IN HUMAN T LYMPHOCYTES

T lymphocytes communicate with their environment through a large array of specialized cell surface receptors. These receptors provide signals which influence T cell ontogeny, activation by antigen, homing and many other aspects of T cell biology. Recent advances have provided a wealth of new information regarding the mechanism by which these surface receptors influence intracellular biochemical events. Transmembrane signaling is initiated by the binding of specific ligand to the extracellular portion of cell surface receptors. The signal is believed to be transmitted across the plasma membrane via either a conformational change in the receptor, by receptor dimerization (1) or by a change in its association with other proteins (2). This change in the receptor in turn induces changes in the level and activity of critical regulatory proteins and second messenger molecules. Then the signal is transmitted into the nucleus, where it induces changes in transcription-regulatory factors. Finally, changes in the activity of transcription factors induce changes in gene expression, leading to the functional and phenotypic markers associated with engagement of that receptor. In this chapter, we will review current knowledge of the molecular events which transpire between engagement of cell surface receptors and transmission of the signal into the nucleus. A summary of current knowledge of the subsequent transcriptional events is beyond the scope of this chapter but may be obtained from recent reviews (3, 4).

New Concepts in Immunodeficiency Diseases. Edited by S. Gupta and C. Griscelli

How can an understanding of signal transduction aid in our understanding of T lymphocyte function? One way is to help to explain those instances where stimulation of different T cell populations in identical ways leads to markedly different outcomes. This situation has been observed repeatedly. First, T cells differ in their responsiveness at different stages of development. T cells at different stages of thymic ontogeny respond differently to binding of the T cell antigen receptor (TCR) by monoclonal antibodies. Only the more mature subset increases intracellular Ca^{2+} in response to antibodies directed against the antigen-binding chains (5). Human cord blood T cells respond in a markedly different way to pharmacological mitogens than adult T cells *in vitro* (6). Second, different clones and subsets of mature T cells have been defined which respond differently to engagement of the TCR. *In vitro* derived T helper cell clones differ markedly in the second messenger pathways activated in response to engagement of the TCR (7, 8). Another example concerns naive and memory T cells, which constitute two readily distinguishable subsets of mature T cells differing in their surface markers and prior exposure to antigen (9). Recent studies have demonstrated that in spite of their equivalent surface expression of TCR, the naive subset is markedly impaired in its ability to proliferate and secrete lymphokines in response to cross-linking of either TCR or CD2 (10, 11). Finally, certain cell populations rendered non-responsive by exposure to specific antigen either *in vivo* or *in vitro* are impaired in their ability to increase intracellular Ca^{2+} and produce lymphokines in response to TCR engagement (12, 13). In each of these examples, T lymphocytes which exhibit comparable levels of surface TCR differ markedly in their response to TCR engagement. Studies of the signal transduction events occurring in these cells can help to determine what accounts for the observed differences. Under different circumstances, differences in responsiveness might occur at the level of the receptor, the signals produced by receptor engagement, or the response of the gene transcription elements to the signals produced.

Another important contribution of signal transduction studies is in elucidating T cell responses to multiple stimuli. During a T cell response to antigen, multiple receptors besides the TCR are engaged. The accessory molecules CD4, CD8, CD45, adhesion molecules and others contribute in important ways to the final outcome. In many cases, accessory molecules play a dual role in immune responses both by promoting cell–cell adhesion upon engagement of their specific ligand on the antigen-presenting cell and by generating an intracellular biochemical signal. Only by defining the nature of the signals they produce and how these signals interact with others generated by the TCR can the role of accessory molecules be fully understood. Accessory molecules play a major role in thymic selection of T cells and in providing the co-stimulatory signals required for antigenic responses of mature T cells. Signal transduction studies can help to sort out how the

cell integrates information entering simultaneously through multiple surface receptors.

T CELL RECEPTOR SIGNAL TRANSDUCTION

Structure and Function of the T Cell Receptor

The T cell receptor for antigen (TCR) is a multi-chain glycoprotein complex (Figure 5.1) which mediates recognition of peptide antigen in association with products of the major histocompatibility complex (MHC) (14, 15). It consists of three components: (a) the clonotypic heterodimeric Ti-α and -β chains which bind specific antigen; (b) the CD3 chains γ, δ and ε; and (c) a disulfide-linked dimer consisting of the ζ chain (16) or members of the ζ family of proteins (17, 18). Ti-α and -β chains are members of the immunoglobulin super-gene family. They have a long extracellular domain which binds specific antigen in association with MHC molecules, and a short intracellular domain which is probably not involved in signal transduction. In contrast, the CD3 chains and ζ chain have relatively short extracellular domains and long cytosolic tails which mediate signal transduction. The CD3 chains are members of the immunoglobulin super-family. The genes encoding these proteins are homologous and are clustered together on chromosome 11, indicating derivation from a common ancestral gene (19, 20). The ζ chain is a member of a small family of related proteins. It is not a member of the immunoglobulin gene family (21, 22). The gene encoding the ζ chain is on chromosome 1 and is not related to the CD3 chains. The other known members of the ζ family are the η chain and the γ subunit of the Fc receptor for IgE (18, 23). The η chain is an alternatively spliced product derived from the same gene as ζ but differing in the C-terminal region (17, 24).

There is evidence for considerable variability in the composition of the TCR. While the Ti-α and -β chains are the antigen-binding chains on most T cells, Ti-γ and Ti-δ are found on an important subpopulation (25, 26). The composition of CD3 chains in the receptor is complex. Evidence from several studies indicates that these chains are not found as a triad. Rather, the CD3-ε chain is associated in a pairwise manner with either CD3-δ or -γ, with two CD3-ε chains per receptor (27, 28). There are also indications that there may be heterogeneity in the composition of the CD3 complex in a single cell in that CD3-ε may be expressed with either CD3-δ or -γ but not both chains in the same complex (29). Finally, TCRs are found with variable composition of ζ family members. Commonly, there is a disulfide-linked ζ–ζ homodimer. However, in murine T cells and possibly in human as well, a fraction (~ 10%) of surface TCRs contain the ζ–η heterodimer (17). Finally, in certain cells, e.g. CTLL, ζ is found associated with the γ chain of the Fc receptor for IgE (18). All of these ζ family dimers are competent for signaling (30, 18). The

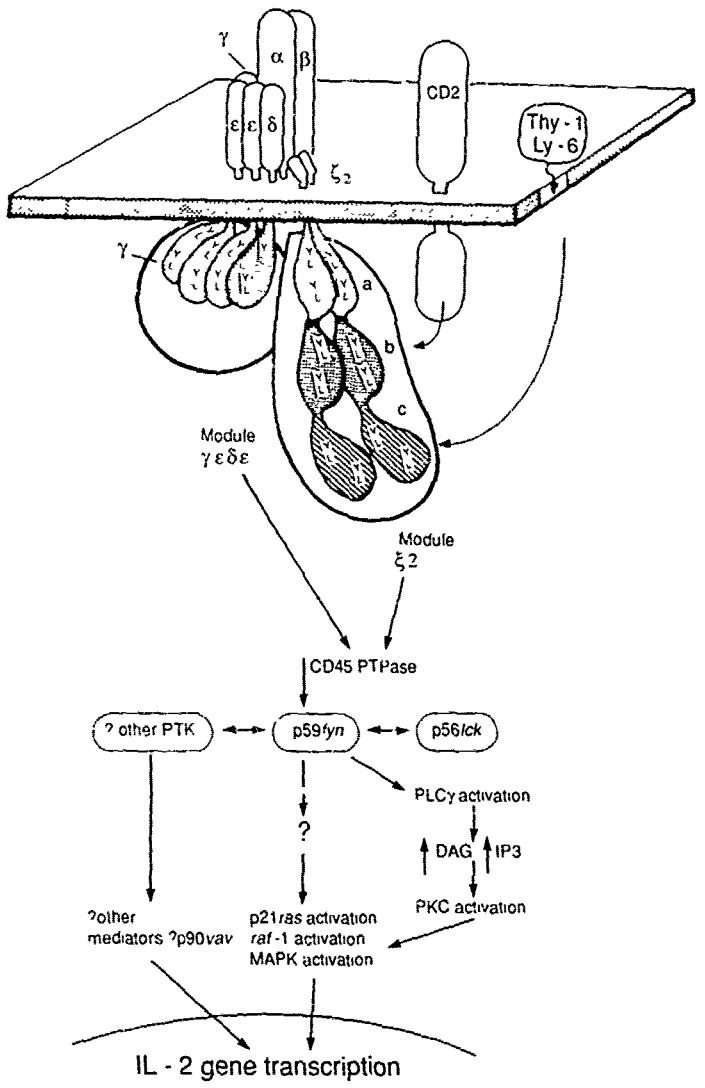

Figure 5.1. Initial events in T cell activation. Activation of either of two distinct modules can initiate signal transduction through the TCR, leading to IL-2 production. The first module consists of the CD3 chains γ, δ and ε, while the other consists of a dimer of chains of the TCR ζ family of proteins. The TCR initiates signal transduction by activating tyrosine kinase activity in a CD45 tyrosine phosphatase-dependent manner. As indicated, p21^{ras}, *raf-1* and MAP kinase have all been implicated in mediating the subsequent transcriptional events. While PKC has been shown to activate each of these mediators, other activators may be involved as well. See text for details. A portion of this figure is modified from Wegener *et al.* (34), with permission

plasticity in the composition of the TCR components provides the potential for considerable variability in signal transduction through the receptor both in different stages of T cell ontogeny and in different subsets of mature T cells.

Which components of the TCR are responsible for signal transduction? As mentioned above, the antigen-binding α/β chains have short cytosolic tails, making them unlikely candidates. In contrast, both the CD3 chains and the ζ chain have long cytosolic tails which could serve to couple to intracellular signaling pathways. To study the signaling capabilities of these chains independent of the rest of the TCR, chimeric molecules have been constructed by several groups wherein the cytosolic tails have been expressed linked to the extracellular and transmembrane domain of an unrelated cell surface protein. These experiments have indicated that both the cytosolic tail of the ζ chain as well as that of CD3-ε are capable of generating a transmembrane signal alone, independent of the other TCR chains (31–33). Furthermore, the quality of the signal appears to be identical to that generated through the whole TCR. All components of signal transduction are seen, including tyrosine phosphorylation, increases in intracellular Ca^{2+}, phosphatidyl inositol (PI) turnover and interleukin 2 (IL-2) production. This finding demonstrates an unexpected redundancy in the structure of the TCR, with multiple components capable of generating a signal. However, there are indications from studies with murine T cells that CD3 chains and ζ chains may transduce different signals in intact T cells. These studies demonstrate that while ζ-deficient and ζ-containing TCR complexes can both signal in response to antigen, only TCR complexes containing ζ chains can support signaling through the accessory molecules Thy-1 and Ly-6 (34) (illustrated in Figure 5.1).

A major advance in understanding how the TCR transmits its intracellular signal came about when several groups determined the sequence motifs in the CD3 chains and the ζ chain which are responsible for signaling. Experiments dissecting the cytosolic tail of ζ have shown that there is an 18-amino acid stretch with two tyrosine residues capable of generating a signal when expressed linked to heterologous proteins (34, 35). This short stretch defines a consensus sequence which is found three times in the cytosolic tail of ζ. It is also found in the cytosolic tails of CD3-γ, -δ and -ε, as well in the immunoglobulin-associated mB-1 and B29 chains and the Fc-ε-associated β and γ chains. The broad distribution of the ζ consensus sequence suggests that it may represent a common mechanism used by a variety of immune system receptors to couple to signal transduction pathways (36).

T Cell Receptor Signaling Pathways

Engagement of the T cell receptor for antigen activates two intracellular signal transduction pathways (Figure 5.1) (37–40). A tyrosine kinase is

activated which phosphorylates the ζ chain of the TCR as well as a large number of other cellular substrates. In addition, phospholipase C (PLC) is activated, which increases PI turnover, leading to increases in diacylglycerol (DAG) and inositil 1,4,5 trisphosphate (Ins(1,4,5)P3) (41). Ins(1,4,5)P3 in turn acts to increase intracellular Ca^{2+}, while DAG activates the serine/threonine kinase protein kinase C (PKC). Like the tyrosine kinase which is activated, PKC phosphorylates components of the TCR—in this case CD3-γ and -ε—as well as a number of other cellular substrates.

The existence of two signal transduction pathways activated through the TCR raises the question of the relationship between the two. Are these two pathways activated independently or is one pathway dependent on the other? Several lines of evidence indicate that they are not independent and that a tyrosine kinase is responsible for activation of the PLC pathway. First, tyrosine phosphorylation can be induced through the TCR in cells in which PKC has been down-regulated pharmacologically, indicating that tyrosine kinase activation is not dependent on PKC (37). Second, anti-phosphotyrosine immunoblotting studies show that tyrosine phosphorylation of substrates can be measured as early as 5–15 s after TCR engagement by monoclonal antibodies (42). This rapid kinetics is more rapid than the generation of Ins(1,4,5)P3 or increases in intracellular Ca^{2+}, suggesting that tyrosine kinase activation precedes PLC activation. Third, pharmacological studies using the selective tyrosine kinase inhibitor herbimycin A demonstrate that tyrosine phosphorylation is required for activation of PLC through the TCR (43). Furthermore, herbimycin A also prevented IL-2 production and expression of high-affinity IL-2 receptors on T cells stimulated through the TCR. Similar results have been obtained with the tyrosine kinase inhibitor genistein (43a). These studies indicate that tyrosine kinase activation is a necessary event for effective signaling through the TCR and that PLC activation is downstream.

Tyrosine Kinase Pathway

The induction of tyrosine phosphorylation following TCR stimulation can best be understood as the end result of two competing enzymatic processes. The level of tyrosine phosphorylation of cellular proteins at any point in time is determined by the rate of phosphorylation by tyrosine kinases, balanced by the rate of dephosphorylation by tyrosine phosphatases (44). Both of these processes appear to be important in regulating tyrosine phosphorylation in T lymphocytes.

What is the TCR-activated tyrosine kinase? While the specific kinase is not known with certainty, several candidates have been proposed which are likely to play an important role in TCR signaling. First of all, unlike the ligand-activated tyrosine kinase growth factor receptors like platelet-

derived growth factor receptor (PDGF-R) or epidermal growth factor receptor (EGF-R), none of the chains of the TCR shares the consensus sequence characteristic of all of the known tyrosine kinases. For this reason, it is likely that the TCR-activated tyrosine kinase is a member of a different class of tyrosine kinase, possibly the $p60^{src}$-related family of cytosolic tyrosine kinases. Several $p60^{src}$-related tyrosine kinases are expressed in hematopoietic cells in general and T-lymphocytes in particular, including $p62^{yes}$, $p56^{lck}$ and $p59^{fyn}$ (45). There is evidence for the involvement of both $p56^{lck}$ and $p59^{fyn}$ in TCR signaling.

The cytosolic tyrosine kinase $p56^{lck}$ is present in T cells in large part associated with the surface receptors of CD4 and CD8 (46–49). This association is mediated by short segments of the cytosolic tail of CD4 and CD8 which have several shared amino acid residues, including two conserved cysteines, a proline and a lysine. On the $p56^{lck}$ molecule, the region responsible for the association is in the N-terminus and includes two critical cysteine residues. It has been postulated that the cysteine residues on CD4/CD8 and $p56^{lck}$ may form a linkage via a metal ion, as has been found in the HIV-encoded tat protein (50). Several lines of evidence suggest a role for $p56^{lck}$ in TCR-mediated signaling. The ligands for CD4 and CD8 are invariant regions of class II and class I MHC molecules, respectively. Thus, while CD4 and CD8 are not linked to the TCR complex in resting cells, they have the potential to become associated with the TCR when it is engaged by a complex of antigen and MHC on the surface of an antigen-presenting cell. Indeed the association of CD4 and $p56^{lck}$ is essential for antigen-specific signal transduction in certain T cells (50a). In addition, a physical association between the TCR and CD4 has been demonstrated to occur in response to TCR engagement even in the absence of antigen-presenting cells (51–54). On cross-linking of either CD4 or CD8, it has been shown that $p56^{lck}$ autophosphorylates and acquires tyrosine kinase activity toward exogenous substrates (47). Another potential mechanism by which the CD4/$p56^{lck}$ and CD8/$p56^{lck}$ complex may signal is suggested by the finding that it is specifically associated with a 32 kDa GTP-binding protein (54a). To determine what role $p56^{lck}$ might play in TCR signalling if it is activated, Abraham *et al.* transfected T cell lines with a constitutively active form of $p56^{lck}$ (55). They found that while active $p56^{lck}$ induced little tyrosine phosphorylation by itself, in combination with TCR stimulation there was strong synergy in tyrosine phosphorylation of substrates and synergy in the resulting IL-2 production. A major role for $p56^{lck}$ in signal transduction events involved in T cell development is indicated by the profound deficits in thymic maturation in mice where the endogenous $p56^{lck}$ gene was knocked out by homologous recombination (56). While these studies indicate that $p56^{lck}$ kinase activation undoubtedly plays a major role in TCR-mediated signaling, other studies indicate a role for other tyrosine kinases as well. In certain murine T

cell lines, TCR is expressed in the absence of surface CD4 or CD8. Yet, in these cells, TCR cross-linking still activates tyrosine phosphorylation and IL-2 production. Furthermore, in the C8 T cell line, cross-linking of CD4 activates $p56^{lck}$ but cross-linking the TCR does not (47). Thus $p56^{lck}$ cannot be the sole tyrosine kinase involved in TCR signaling.

Samelson *et al.* demonstrated the existence of a tyrosine kinase interacting directly with the TCR by co-precipitating tyrosine kinase activity with the receptor (57). Using specific antibodies they determined that the cytosolic tyrosine kinase $p59^{fyn}$ co-precipitated with the receptor, while $p62^{yes}$ and $p56^{lck}$ did not. They were unable to demonstrate an increase in $p59^{fyn}$ kinase activity in response to TCR stimulation. However, the inability to detect a change in kinase activity may be technical in nature, possibly reflecting a detergent-induced activation of $p59^{fyn}$ in the resting cells. An important role for $p59^{fyn}$ in T cell function is further suggested by the fact that alternate splicing of one of the exons of $p59^{fyn}$ gives rise to a unique form of this kinase in T cells compared to other tissues where it is expressed (58). Direct evidence for a potential role for $p59^{fyn}$ in TCR-mediated signaling is provided by transgenic studies wherein $p59^{fyn}$ is over-expressed in thymocytes (59). In normal mice, immature CD4+CD8+ thymocytes are characterized by ten-fold lower levels of $p59^{fyn}$ compared to mature thymocytes, and depressed responsiveness to TCR stimulation as measured by Ca^{2+} mobilization. By contrast, in the transgenic mice, $p59^{fyn}$ expression by the immature thymocytes and TCR-stimulated Ca^{2+} mobilization are both increased to the same levels seen in the mature population. While these studies suggest an important role for $p59^{fyn}$ in TCR signaling, there are indications that it is not the only TCR-associated tyrosine kinase. Experiments in the Jurkat human T cell line have demonstrated a TCR-associated tyrosine kinase activity which is not $p59^{fyn}$ (60). This kinase activity is observed specifically in stimulated cells and is selectively associated with the ζ chain of the TCR. Taken together, these studies show that while $p59^{fyn}$ can clearly associate with the TCR, other tyrosine kinases can as well. Further experiments will be required to determine whether several different cytosolic tyrosine kinases including $p59^{fyn}$ can serve as the TCR-stimulated tyrosine kinase or whether another as yet undefined tyrosine kinase acts as the common signal-transducing kinase.

Interest in the role of tyrosine phosphatases in TCR-mediated signaling was first kindled by the discovery that the highly expressed cell surface glycoprotein CD45 is a tyrosine phosphatase, as revealed by the presence of a tandemly repeated tyrosine phosphatase domain in its cytosolic tail (61). Since that time, it has been shown that CD45 is a member of a large family of transmembrane and cytosolic tyrosine phosphatases, several members of which are expressed in T lymphocytes (62). The importance of CD45 in T cell activation was shown by the finding that certain CD45– T cell lines are

incapable of proliferating in response to antigenic stimulation or cross-linking of the antigen receptor (63). However, proliferation in response to exogenously added IL-2 was intact. Koretzky *et al.* demonstrated that early signaling events are also dependent on CD45 since CD45– HPB–ALL cells did not increase intracellular Ca^{2+}, PI turnover, tyrosine phosphorylation or IL-2 production in response to engagement of the TCR (64, 65). TCR-mediated tyrosine phosphorylation is also impaired by treatment of cells with the tyrosine phosphatase inhibitor phenyl arsine oxide (PAO) (66). These studies indicate that CD45 may be involved in the very earliest events in the TCR signal transduction cascade, perhaps by regulating events required for tyrosine kinase activation.

How does CD45 function in T cell activation? One approach to understanding its function is to identify tyrosine-phosphorylated substrates. Studies cross-linking CD45 with surface activation receptors have suggested some potential substrates for CD45. The physiological relevance of these substrates is suggested by the following studies. Cross-linking of CD2 or CD3 on T lymphocytes induces T cell activation. However, if these molecules are co-aggregated with CD45, activation is inhibited as measured by the failure to increase cytosolic Ca^{2+} or IP_3 levels (42, 67). Interestingly, this CD45-mediated inhibition is correlated selectively with impairment in tyrosine phosphorylation of a 100 kDa protein substrate on anti-phosphotyrosine blotting. This 100 kDa phosphoprotein may thus represent a substrate for CD45. Alternatively, CD45 co-aggregation may inhibit activation of a tyrosine kinase which phosphorylates this protein. The requirement for the expression of CD45 to induce tyrosine kinase activation may be explained by its ability to dephosphorylate an inhibitory tyrosine phosphorylation site on $p56^{lck}$ at residue 505 and possibly on other $p56^{lck}$-related kinases such as $p59^{fyn}$ (68, 69). In one study of three independently derived pairs of CD45– and CD45+ murine T cell lymphomas, the CD45-expressing cells were consistently deficient in phosphorylation of $p56^{lck}$ at Tyr-505. Furthermore, co-cross-linking CD4 with CD45 induces dephosphorylation of $p56^{lck}$ at Tyr-505 (69). Clearly, CD45 is capable of influencing the state of phosphorylation and activity of $p56^{lck}$ *in vivo*. A determination of the other *in vivo* substrates of CD45 will help to further define the role of this key regulatory molecule.

Tyrosine Kinase Substrates

Defining the tyrosine phosphorylated substrates will be critical for understanding how tyrosine kinases mediate TCR signaling. Antiphosphotyrosine blotting demonstrates upwards of ten cellular proteins whose tyrosine phosphorylation is increased significantly in response to TCR cross-linking. Micro-sequencing of proteins identified by binding to antiphosphotyrosine

antibodies is currently being used to determine the identity of these substrates (39). However, some substrates may be tyrosine phosphorylated at a level too low to detect in antiphosphotyrosine blots of whole cell lysates. Another approach which has been highly productive for defining substrates has been to look for tyrosine phosphorylation of enzymes and other proteins suspected to be involved in signal transduction. Fibroblasts stimulated through tyrosine kinase growth factor receptors has been a particularly fruitful system for discovering significant tyrosine-phosphorylated substrates. Some of these same substrates have been shown to be tyrosine phosphorylated in T cells as well. A list of the currently identified tyrosine-phosphorylated substrates is provided in Table 5.1.

Table 5.1 Identified tyrosine-phosphorylated substrates in T cell activation

Stimulus	Substrate	Potential function	Ref.
TCR stimulation	$PLC_\gamma 1$	Catalyzes PI hydrolysis	70–72
	$p90^{vav}$	Mediates transcriptional activation	76, 77
	ezrin	?Mediates cytoskeletal rearrangement	39
	TCR_ζ	Activates signal transduction	16
	CD45	Modulates tyrosine dephosphorylation	79
IL-2 stimulation	PI-3K	?Promotes proliferation	212
	$IL\text{-}2R_\beta$	Activates signal transduction	210, 220
	$p56^{lck}$	Activates tyrosine phosphorylation	222
	raf-1	Activates serine/threonine phosphorylation	213

One of the most important tyrosine-phosphorylated substrates following TCR stimulation is phospholipase C (70–72) (Table 5.1). Several isoforms of PLC exist in cells but only the γ isoforms have SH2 (*src*-homology region 2) and SH3 domains (see below) Following TCR engagement, the γ isoform of PLC is rapidly tyrosine phosphorylated in a time course which parallels activation of PLC activity *in vivo*. Tyrosine phosphorylation of PLC_γ has been demonstrated *in vitro* to activate its enzymatic activity (73–75). These observations complement the finding that tyrosine kinase activity is required for activation of the PLC pathway of TCR signaling and support the hypothesis that tyrosine kinase activation is indeed a primary event.

A newly defined tyrosine-phosphorylated substrate which may provide insight regarding transmission of signals into the nucleus is the proto-oncogene product $p95^{vav}$ (76, 77). This protein is expressed selectively in hematopoietic cells. On cross-linking of CD3 with CD4, T lymphocytes exhibit tyrosine phosphorylation of $p95^{vav}$ as rapidly as 30 s after stimulation. The predicted amino acid sequence of $p95^{vav}$ reveals several interesting properties. It has an SH2 domain and two SH3 domains (78). These sequences allow

specific association with tyrosine-phosphorylated proteins and are found on many tyrosine-phosphorylated substrates, including PLC_γ. In addition, $p95^{vav}$ has nuclear localization signals and sequences characteristic of transcription factors, including a leucine zipper domain and a helix–loop–helix domain. These features suggest that $p95^{vav}$ may localize to the nucleus after TCR/CD4 stimulation, where it may modulate gene transcription.

Another protein which has been shown to be a possible tyrosine kinase substrate after TCR stimulation is CD45 (79). CD45 tyrosine phosphorylation was transient. If tyrosine phosphorylation of CD45 has effects on its function, this observation may demonstrate cross-talk between tyrosine kinase and tyrosine phosphatase components involved in early signal transduction. Several other important signaling molecules have been reported to be tyrosine phosphorylated in other cell types but not yet in T cells stimulated through the TCR, in particular a set of SH2-containing proteins which includes: c-*crk*; the 85 kDa subunit of phosphatidyl inositol 3-kinase; the $p21^{ras}$-GTPase activating protein (*ras*-GAP); and the 62 and 190 kDa GAP-associated proteins (80). In addition, a newly described protein tyrosine phosphatase (81) also has an SH2 domain, although it has not yet been shown to be tyrosine phosphorylated.

Phospholipase C Pathway

Activation of PLC is an early event following TCR stimulation. It acts to hydrolyze PIP2 (phosphatidyl-inositolbisphosphate) to IP3 and diacylglycerol IP3 is a hydrophilic cytosolic mediator which binds to specific receptors located in the endoplasmic reticulum (82), opening channels which release stores of Ca^{2+} into the cytosol. Increases in cytosolic Ca^{2+} are also derived from the extracellular medium. The mechanism underlying this latter Ca^{2+} flux is still controversial. In combination with Ca^{2+}, diacylglycerol binds to and activates PKC. The events subsequent to PKC activation are not known in detail because few substrates of PKC have been defined with certainty. Further complexity is added by the fact that PKC is not a single enzyme but a family of related isoforms (83). Different isoforms of PKC are activated differently and have different subcellular localization (84). PKC is known to phosphorylate the CD3-γ and -ε chains of CD3 (85), but the effects of this phosphorylation are not known. It is known, however, that PKC activation induces the AP-1 transcription factor which consists of c-*fos* and c-*jun*. Binding sites for AP-1 are present in the promoter region of a variety of different genes, including the IL-2 gene (4). AP-1 is also a component of NFAT (nuclearfactor of activated T cells), a transcription factor present in activated T cells, which binds to the promoter region of the IL-2 gene (86). Thus activation of PKC by PLC has the potential to induce new gene expression, and is likely to contribute to IL-2 gene expression.

While much evidence supports the model that PKC activation is responsible for induction of IL-2 gene expression following TCR stimulation, there is evidence that other mechanisms may be involved. Supporting a role for PKC are experiments which have shown that pharmacological activation of PKC by phorbol ester in combination with calcium ionophores is a sufficient stimulus for IL-2 production (41). In addition, recent experiments have demonstrated that activation of the PLC pathway in Jurkat cells through a transfected G-protein-coupled receptor activates IL-2 production by itself without activation of tyrosine kinases (87). However, other experiments suggest that pathways not involving PKC may also induce IL-2 production. First, in certain cells, TCR engagement has been observed to induce IL-2 production without any evidence of PI hydrolysis or any increase in intracellular Ca^{2+}, therefore suggesting an absence of PLC activation (88). In addition, gene transfection experiments have suggested that tyrosine kinases may induce IL-2 production by themselves. Specifically, transfection of T cell hybridoma cells with an active cytosolic tyrosine kinase ($p60^{c\text{-}src}$) led to constitutive IL-2 production in a dose-dependent manner (89). Nonetheless, there was no evidence of PKC activation. These studies demonstrate that tyrosine phosphorylation may contribute to IL-2 production by pathways which do not involve PKC.

Other Mediators

A variety of other signal transduction mediators have also been implicated in TCR signaling. Some of these—including the GTP-binding protein $p21^{ras}$ and the serine/threonine kinases *raf*-1, MAP kinase and $p90^{rsk}$ kinase—were first found to be involved in signaling through tyrosine kinase growth factor receptors and subsequently found to be activated in T cells as well.

The protooncogene product *ras* is a 21 kDa monomeric GTP-binding protein which is found in mutated form in a large variety of transformed cells (90). It has been shown to have growth-promoting properties, as shown by its ability to transform cells *in vitro* when mutated forms are transfected in combination with other oncogenes. Inactive $p21^{ras}$, found in resting cells, is bound to GDP, while active $p21^{ras}$ binds GTP; $p21^{ras}$ has an intrinsic GTPase activity which hydrolyzes the bound GTP, rendering the protein inactive. To determine whether $p21^{ras}$ was activated in response to TCR stimulation, Downward *et al.*, compared levels of GTP- and GDP-bound $p21^{ras}$ in resting and activated T cells (91). They found that TCR stimulation led to a substantial, rapid increase in the proportion of $p21^{ras}$ in the GTP-bound, active form. Increases in GTP-bound $p21^{ras}$ can occur by either of two mechanisms: there can be an increase in the guanine nucleotide exchange rate, thereby substituting the more abundant GTP for GDP; alternatively, there can be a decrease in the intrinsic GTPase activity of $p21^{ras}$, leading to stabilization of the GTP-

bound form. Cellular enzymes have been characterized which mediate both these activities: guanine nucleotide exchange factors (92) and GAP enzymatic activities, respectively. Downward *et al.* determined that increases in GTP-bound $p21^{ras}$ following TCR stimulation occurred because of a decrease in GAP activity in the cell with no change in the rate of nucleotide exchange. The proteins *ras*-GAP (93, 94) and NF1 (95–97) have both been shown to regulate the intrinsic GTPase activity of $p21^{ras}$. However, it is not currently known what protein is responsible for regulating *ras* GTPase following TCR stimulation. Pharmacological activation of PKC also activates $p21^{ras}$ and decreases cellular activation, but it is not known whether this is the only mechanism responsible for the effects of TCR stimulation on *ras* activation (98).

The demonstration that $p21^{ras}$ is activated when the TCR is engaged suggests that it may serve to mediate some of the transcriptional effects of TCR stimulation. Support for this possibility is provided by studies of Baldari *et al.*, who introduced constitutively active $p21^{ras}$ into murine EL-4 T cells (99). Ordinarily these cells require calcium ionophore and phorbol ester for IL-2 production. While $p21^{ras}$ alone did not induce IL-2 gene expression, it did substitute for phorbol ester, allowing IL-2 gene expression with calcium ionophore alone. IL-2 production by these transfected cells in response to calcium ionophore was not inhibited by the PKC inhibitor H7. While the effects of H7 are not completely specific for PKC, these results suggest that $p21^{ras}$ activation is upstream of PKC in the sequence of events leading to new gene transcription.

The cytosolic protein *raf-1* (also known as c-*raf*) is a cytosolic serine/threonine kinase which is activated within minutes of TCR stimulation (100). *raf-1* has been shown to occupy a key position in signal transduction through many different growth factor receptors (101–104). Down-regulation of *raf-1* protein by anti-sense mRNA expression prevents proliferation of fibro blasts in response to serum or purified growth factors (105). In addition, expression of constitutively active *raf-1* has been shown to induce expression of a variety of new genes, including c-fos (106) and a member of the jun gene family (107), showing that receptor-mediated activation of *raf-1* may influence transcriptional events. In murine T lymphocytes, TCR stimulation induces hyperphosphorylation of a majority of the *raf-1* present in the cell. The enzymatic activity of *raf-1* also increases with TCR stimulation as measured *in vitro* by phosphorylation of both endogenous and exogenous substrates. The pathway of activation of *raf-1* through the TCR has been shown to depend on the activation of PKC, as it is abolished in PKC-depleted cells. In this respect, the TCR behaves differently from growth factor receptors where *raf-1* activation is independent of PKC (108). In human T cells, *raf-1* activation occurs rapidly, reaching peak levels as early as 30 s after TCR cross-linking by anti-CD3 monoclo nal antibodies (39).

Nonetheless, it requires activation of both a tyrosine kinase and a tyrosine phosphatase, demonstrating that *raf-1* activation is downstream of these other signal transduction mediators. These observations suggest that the early events following TCR stimulation are coordinated in a highly efficient manner which allows coupling of the receptor to *raf-1* through a series of intermediate events with minimal delay. An important key to determining the role of *raf-1* will be to define its substrates *in vivo*. In view of the known effects of *raf-1* on gene transcription and the observed translocation of *raf-1* to the nucleus (109, 110), it is likely that *raf-1* activation mediates some of the transcription events following TCR stimulation, perhaps by phosphorylating nuclear factors involved in gene expression.

The study of signal transduction pathways activated following stimulation of tyrosine kinase growth factor receptors has revealed the existence of a protein kinase cascade wherein one protein kinase phosphorylates and activates another, thereby propagating the signal. Among the known growth factor-activated protein kinases are the serine/threonine kinases MAP kinase and $p90^{rsk}$. These kinases have also been shown to be activated in response to TCR stimulation (111). MAP kinase was first named for its ability to phosphorylate microtubule-associated protein-2 (112). MAP kinase is believed to phosphorylate and activate the serine/threonine kinase $p90^{rsk}$ *in vivo*, supporting the likelihood of a protein kinase cascade. *In vitro* data suggests it may also phosphorylate a range of other substrates, including the transcription factors myc and jun; however, further experiments are required to assess whether it has the same effect *in vivo*. MAP kinase has been shown to be activated by phosphorylation on both tyrosine and threonine residues (113, 114). Enzyme activities capable of catalyzing these phosphorylation events have been isolated from activated cells, but the identity of the proteins involved has not yet been determined (112, 115, 116). In T lymphocytes, MAP kinase has been shown to be activated in response to cross-linking of both CD3 and CD2 (117, 118). Phorbol ester stimulation also activates MAP kinase, suggesting that the PLC–PKC pathway may be involved. However, depletion of PKC only partially inhibits activation of MAP kinase, raising the possibility that a PKC-independent pathway may participate as well. Studies of Ettehadieh *et al.* suggest that the CD4/$p56^{lck}$ complex may be involved, since they found that cross-linking of CD3 molecules activated MAP kinase in a CD4+ T cell line but not in a matched CD4-non-expressing line (119). MAP kinase also serves as a substrate for $p56^{lck}$ *in vitro*; however, it has not been demonstrated that $p56^{lck}$ phosphorylates MAP kinase directly *in vivo*. In addition, the tyrosine phosphatase CD45 may play a role in turning off MAP kinase after cell activation (117, 120). Thus, tyrosine phosphorylation and dephosphorylation may mediate both the positive and negative regulation of MAP kinase activity.

CYCLOSPORIN A AND FK 506

The immunosuppressants cyclosporin A (CSA), FK 506, and rapamycin have provided substantial insight into later signal transduction events following TCR stimulation (121, 122). Many studies have shown that these agents selectively inhibit calcium-dependent pathways of T cell activation that lead to lymphokine gene transcription (123). These agents are relatively selective for T cells, and their effects are specific for the mode of T cell activation, in that they are ineffective at preventing T cell activation by calcium-independent pathways (124). CSA and FK 506 inhibit essentially identical aspects of T cell activation, while rapamycin has distinct effects. Both CSA and FK 506 efficiently inhibit TCR-induced lymphokine gene transcription, T cell proliferation, and TCR-induced apoptosis (125, 126). In contrast, rapamycin interferes with lymphokine signal transduction, a process not inhibited by CSA or FK 506. Indeed, Sigal and co-workers made the intriguing observation that rapamycin is able to reverse the effects of FK 506 by competitive binding to the same intracellular receptor (127).

Both CSA and FK 506 bind to distinct families of intracellular immunophilin receptors: the cyclophilins and FK binding proteins (FKBP), respectively (128, 129). Cyclophilin and FKBPs are peptidyl-prolyl *cis–trans* isomerases (rotamases) that are expressed at abundant levels in many tissues, and are thought to be involved in the assembly of newly translated proteins (130). CSA inhibits the rotamase activity of cyclophilin, although it has never been clear as to how this would confer the relatively specific effects of CSA on lymphokine gene transcription. Several lines of evidence indicate that CSA and FK 506 do not function simply by inhibiting the function of their respective intracellular immunophilin receptors. For example, analogs of CSA exist that bind to and inhibit rotamase activity of cyclophilin, and yet are not immunosuppressive (131). Thus, rotamase inhibition cannot explain the immunosuppressive effects of these agents.

A current model to explain the immunosuppressive effects of CSA on T cell activation is shown in Figure 5.2. Calcineurin is a calcium- and calmodulin-dependent serine/threonine phosphatase that is expressed in the brain and in T lymphocytes. Recently, it was found that both cyclophilin and FKBPs can bind to calcineurin, forming a ternary complex of immunosuppressant, immunophilin, and calmodulin (132, 133). Thus, the effector complex potentially consists of at least six components, as calcineurin consists of a 61 kDa A subunit, containing the catalytic phosphatase site and a binding site for calmodulin, and a regulatory 19 kDa B subunit, containing a calcium binding site (Figure 5.2). The model to explain the effects of FK 506 is essentially the same, except that FK 506 would encounter calcineurin via an FKBP. This model is likely to become even more complex. For example,

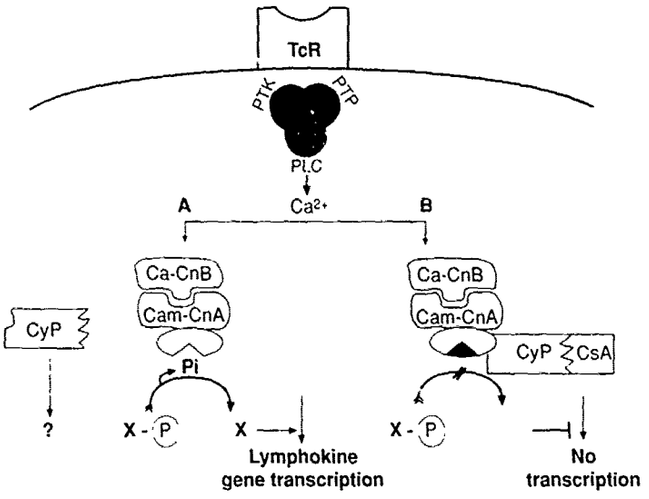

Figure 5.2. Model of cyclosporin A-mediated immunosuppression. The interaction of the TCR with antigenic ligand on APC results in protein tyrosine kinase (PTK), protein tyrosine phosphatase (PTP) and phospholipase C (PLC) activity, generating increased $[Ca^{2+}]i$. (A) In the absence of cyclosporin A (CsA), calcium interacts with calcineurin B (CnB) and forms a complex with calmodulin and calcineurin A (Cam–CnA). Calcineurin phosphatase activity results in dephosphorylation of protein X, which is required for the induction of cytokine gene transcription. The physiological function of the rotamase cyclophilin (CyP) is unknown (B) In the presence of CsA, CsA binds to CyP, and the resulting complex binds to the calcineurin (Ca^{2+}–CnB–Cam–CnA) complex, inhibiting phosphatase activity. Protein X remains phosphorylated, and cytokine gene transcription is prevented

both cyclophilin and FK 506 belong to distinct families of proteins, of which new members are continually being appreciated (133, 134).

Both CSA and FK 506 inhibit calcineurin phosphatase activity (135). The inhibition of cellular calcineurin phosphatase activity occurs at similar or identical concentration of CSA or FK 506 to those required to inhibit cellular IL-2 production (135). Thus, calcineurin appears to be the relevant intracellular target of CSA and FK 506. Furthermore, these findings indicate that the calcineurin phosphatase is likely to play a crucial role in signal transduction in T cell activation (136). Finally, the physiological functions of immunophilins in the absence of immunosuppressants remains unknown.

CO-STIMULATORY RECEPTORS

Normal T cells or T cell clones do not proliferate in response to TCR ligation by antigen unless other signals are provided by antigen-presenting cells

(APC) (13). In most cases, the co-stimulatory signal provided by the APC requires cell contact, while in some instances soluble factors have been shown to provide an efficient co-stimulatory signal. For example, in the mouse, interleukin 1 (IL-1) produced by APC appears to provide a co-stimulatory signal to T_H2 clones that produce IL-4 (137). However, soluble factors do not appear to provide the co-stimulatory signals in the case of mouse T_H1 clones that produce IL-2. Most normal human and mouse CD4+ T cells appear to require the interaction of adhesion receptors between the T cell and the APC in order to deliver the co-stimulatory signal, as purified CD4+ T cells or T cell clones do not produce IL-2 when cultured on planar lipid membranes that contain antigen-bearing MHC class II ligands unless APC are also present (13).

Results from several laboratories indicate that the signal provided by the CD28 adhesion receptor is capable of providing a co-stimulatory signal. CD28 is a 44 kDa glycoprotein expressed as a homodimer on the surface of most peripheral blood T cells and thymocytes (138). CD28 is a member of a receptor family that also includes CTLA-4, a molecule that is expressed in activated T cells (139). In both mouse and man, anti-CD28 mAbs have stimulatory effects that greatly enhance IL-2 production and proliferation of T cells stimulated with anti-CD3 mAb or mitogenic lectins (138, 140).

The B7 or BB-1 receptor is a 45 kDa antigen that is expressed on activated B cells and monocytes (141, 142). B7 is the counter-receptor for both the CD28 and CTLA-4 receptors that are expressed on T cells (143, 144). The cross-linking of MHC class II molecules on APC induces B7 expression (145). Transfection of non-lymphoid cells with B7 cDNA confers co-stimulatory properties to the cells in conjunction with anti-CD3 mAb or lectins (146). Alloantigen-induced T cell proliferation can be prevented if the interaction between CD28 and B7 is blocked (145). Further evidence for the role of CD28 and B7 interaction in providing a second signal during T cell activation is derived from observations that the efficiency of APC function correlates with B7 expression: the ability of B cell lymphoma lines to stimulate T cell proliferation in a mixed lymphocyte culture is correlated with the density of B7 expression (147). Furthermore, small B cells that have little or no B7 expression stimulate T cells poorly, and in some instances may even elicit T cell tolerance (148). In contrast, activated B cells display efficient APC function.

The biochemical pathway used by the CD28 receptor remains poorly understood. In resting T cells, ligation of CD28 by mAb does not induce calcium mobilization, in contrast to TCR or CD2 cross-linking (149). Inhibition of protein tyrosine kinase activity by herbimycin A prevents CD28-induced IL-2 production, and ligation of the CD28 receptor by anti-CD28 mAb induces tyrosine phosphorylation in T cells (150). Chinese hamster ovary (CHO) cells transfected with B7 cDNA induce tyrosine

phosphorylation in T cells. CD28-induced tyrosine phosphorylation differs from TCR-induced tyrosine phosphorylation in several aspects. The pattern of substrate tyrosine phosphorylation differs after CD28 and TCR ligation. TCR ligation induces tyrosine phosphorylation of the TCR ζ chain, while CD28 ligation does not. Resting T cells do not respond to CD28 ligation, while resting T cells respond to TCR ligation. In contrast, after activation by anti-TCR or PMA, T cells have a strong response to CD28 ligation.

A central question concerning the role of accessory molecules in T cell activation is whether or not the accessory signal is enhancing the signals provided by the T cell receptor, or whether the signal is distinct from the T cell receptor. Several studies indicate that CD28 functions independently from the T cell receptor during the course of T cell activation. CD28 appears to initiate a signal transduction pathway distinct from the T cell receptor, in part because CD28 stimulation was shown to enhance lymphokine production even in the presence of maximal phorbol ester and calcium ionophore stimulation, signals that mimic those provided by the TCR (151). In addition, increased mRNA levels and secretion of IL-2, interferon-γ, GM-CSF, and TNF-α can all be induced by a combination of PMA and anti-CD28 stimulation (152). This means of producing IL-2 is completely resistant to suppression by cyclosporin A in normal T cells. In contrast, the major transcriptional stimulation of IL-2 mediated by the T cell receptor or phorbol ester plus calcium ionophore can be completely abolished by cyclosporin A.

The primary mechanism by which anti-CD28 augments lymphokine production in mature T cells is by inhibiting the degradation of lymphokine mRNAs (153). As a result of the stabilization of mRNA, the steady state levels of lymphokines increase, leading to enhanced translation and protein secretion. In addition to a primary effect on mRNA stability, co-stimulation of quiescent T cells with anti-CD3 and anti-CD28 does appear to have a number of secondary effects on T cell responses. Late after stimulation, IL-2 mRNA levels appear to be enhanced by a CD28-dependent increase in transcription as well as mRNA stability. This appears to be the major mechanism whereby CD28 stimulation increases lymphokine production in the Jurkat leukemia line (154). Weiss and colleagues have identified a DNA binding protein that binds to a site in the IL-2 promotor that is distinct from the previously described sites and is required for CD28-mediated responses (154). Thus, it appears that CD28 may increase lymphokine production by several mechanisms. The binding site mediating CD28 effects is functional in primary T cells (155) as well as in Jurkat cells; however, the biochemical signal provided by CD28 appears to differ in that the transcriptional increases in IL-2 production are cyclosporin A sensitive in Jurkat cells while in primary T cells they are cyclosporin A resistant (156). It remains to be determined whether this reflects heterogeneity of signal transduction among primary T cells, or whether this is a phenomenon limited to transformed cell lines.

CD28 appears to have other effects beyond its role in lymphokine secretion. The binding of CD28 mAb to T cells induces increased adhesion of several integrin receptors (157), and it is possible that these receptors might induce some of the effects attributed to CD28 stimulation. CD28 stimulation by mAb or by B7-expressing cells induces cytotoxic activity in resting CD8+ T cells (158). It is not yet clear if this is a direct effect of CD28-mediated signal transduction, or if lymphokines produced consequent to CD28 activation might play a role in the cytotoxicity.

OTHER SIGNALING MOLECULES INVOLVED IN T CELL ACTIVATION

There is a growing number of surface molecules other than growth factor receptors that are involved in T cell activation (Table 5.2). The molecules listed in the table are candidate receptors that are currently known or suspected to be involved in T cell activation. Some of the molecules are expressed on activated and resting T cells, while others are found on activated T cells only, so it is likely that some of the molecules have distinct functions that may be limited to certain phases of the cell cycle. For most of the receptors, a ligand has been discovered that is present on accessory cells or

Table 5.2 Cell surface accessory receptors involved in signal transduction and T cell activation

T cell accessory receptor	Counter-receptor[a]	Ref.[b]
CD2	LFA-3 (CD58)	159–168
CD4	MHC class II	46–48, 55, 169, 170
CD8	MHC class I	171–172
CD5 (Ly-1)	CD72 (Lyb-2)	173–175
CD6	?	176, 177
LFA-1 (CD11a/CD18)	ICAM (CD54)	183–186, 192
CD28	B7	140–158
CTLA-4[c]	B7	139
Thy-1[d]	?	187–189
Ly-6[d]	?	190
Heat-stable antigen[d]	?	191
CD69[c]	?	197–200
MHC class II[c]	?CD4	178
CD26	Collagen	180–182
VLA-4[c]	Fibronectin	192, 194, 195
VLA-5[c]	Fibronectin	193, 194
VLA-6[c]	Laminin	192, 195, 196

[a]In some cases the existence of a ligand (counter-receptor) is not known (?).
[b]References selected indicate potential *in vitro* or *in vivo* role of T cell surface molecules in T cell activation
[c]Surface expression in activated T cells and not on resting T cells.
[d]Antigens expressed on mouse T cells

in the extracellular matrix, while in other cases a ligand (soluble or cell bound) is not known to exist, and the function of the molecule on T cells is inferred from the *in vitro* effects of monoclonal antibodies that are presumed to mimic physiological ligands upon binding. In almost all cases it is not yet known if the receptors are involved in physiological T cell activation that occurs *in vivo*. These receptors should also be considered as potential candidates that might be involved in certain forms of pathological T cell activation.

In many cases it is difficult to distinguish whether the molecules function by increasing intracellular adhesion, thereby augmenting or sustaining signals delivered through the TCR, or, alternatively, by transmitting a signal independent of the TCR. Shaw and colleagues have addressed this question by immobilizing ligands for accessory receptors on beads, and asking whether the ligand can deliver a co-stimulatory signal to T cells when immobilized on a different bead, or only when immobilized on the same bead that is coated with anti-CD3 mAb (159). Co-stimulation occurring only when the two ligands are immobilized on the same bead is termed "local co-stimulation" while co-stimulation occurring with the ligands on different beads is termed "remote co-stimulation", and indicates that the accessory signal cannot function simply by increasing adhesion and thereby promoting TCR signaling.

CD2 is an adhesion receptor that is expressed on T cells and NK cells. The physiological role of CD2 remains unknown, and it has been argued that the early expression of CD2 in T cell ontogeny may signify a role in T cell differentiation (160). Treatment of mice with anti-CD2 mAbs *in vivo* is able to induce T cell unresponsiveness (168). There appear to be several counter-receptors for CD2, one of which is CD58 (LFA-3). CD2 is able to activate phospholipase C in T cells and NK cells (160, 164), and it appears that CD2 can increase phospholipase A2 activity (165). It remains controversial as to whether CD2 can function independent of the TCR in T cells; however, most studies indicate that it cannot (162). Antigen-specific T cell responses were found to be augmented in T cells that were transfected with CD2 cDNA that lacked the cytoplasmic tail, suggesting that adhesion rather than signaling is a primary function of CD2 (163). On the other hand, Shaw and co-workers found that CD2 could deliver "remote co-stimulation" (159), implying that a component of the signal delivered by CD2 is independent of adhesion.

CD4 and CD8 function in MHC-restricted antigen presentation by binding to invariant regions of the MHC molecules on APC, and these interactions promote antigen recognition and signaling by the TCR. As discussed above, CD4 transmits signals important for T cell activation via the associated protein tyrosine kinase $p56^{lck}$ (169, 170). CD4 cross-linking increases activity of the *lck* kinase but it does not activate phospholipase C. CD8 is expressed as a homodimer or a heterodimer, and transmits signals after interaction with the counter-receptor MHC class I molecule that is expressed

on APC. The binding of CD8 to MHC class I proteins is increased upon signal transduction through the TCR (172). There is evidence that the signal delivered by CD8-αβ heterodimers differs from that of cells that express CD8-αα homodimers (171).

CD5 is expressed on T cells, and at low levels on some B cells. The counter-receptor for CD5 is CD72, which is found on B cells (173). Cross-linking of CD5 with mAbs co-stimulates with CD3, and activates PI metabolism and tyrosine phosphorylation (174, 175). In some cases CD5 stimulation by particular CD5 mAbs is able to activate protein kinase C in the absence of detectable PI breakdown or calcium mobilization (175). Signal transduction by CD5 appears to require surface expression of the TCR on T cells It is not yet known if the binding of the natural ligand to CD5 also activates T cells. In the mouse, the homologous molecule Ly-1 appears to have similar functional effects

The binding of mAb to LFA-1, or the addition of its purified ligand ICAM-1 to T cells, causes "remote" co-stimulation (159). TCR-mediated signal transduction increases adhesion of T cells, in part, by an increase in the avidity of LFA-1 for ICAM-1 (183). LFA-1 cross-linking on T cells results in increased $[Ca^{2+}]_i$ when CD11a (LFA-1 α) but not when anti-CD18 (LFA-1 β) chain mAbs are used (186) Furthermore, LFA-1 mAbs prolong CD3-induced calcium signals over that observed when CD3 alone is used (185). The administration of mAbs to LFA-1 and ICAM-1 prevents cardiac allograft rejection, and appears to result in the induction of specific tolerance (184). Thus, while the mechanism for this very interesting effect remains to be determined, it is likely that the signal delivered by the interaction between LFA-1 and ICAM-1 provides an important co-stimulatory signal in the context of alloantigen presentation, and may be important in regulating T cell proliferation and tolerance induction.

In the mouse, monoclonal antibodies to Thy-1 or Ly-6 cause potent co-stimulatory effects on T cells, and activate phospholipase C (188–190). Unlike other co-stimulatory molecules, these molecules have a glycosyl PI linkage, and are associated with tyrosine kinases of the *src* family (187). Studies to date indicate that Thy-1 and Ly-6 require the presence of the TCR in order to provide a complete signal, although, as discussed below, these molecules appear to be able to activate programmed cell death in the absence of TCR expression.

Antibodies to CD69 augment CD3-induced proliferation of T cells (197, 198). CD69 is associated with a GTP binding protein (199). While the signal transduction pathway used by CD69 remains unknown, the binding of anti-CD69 increases transcription factor AP-1 activity (200). Heat-stable antigen is a mouse antigen that provides a co-stimulatory effect for CD4+ T cells during antigen-induced activation (191). Ligands for CD69 and heat-stable antigen have not yet been discovered.

MHC class I and class II molecules are expressed on APC and are thought to play an important role in antigen-mediated T cell activation via their associated counter-receptors on T cells, CD8 and CD4. MHC class I products are expressed on all T cells, while activated human T cells express MHC class II, and evidence is accumulating that these molecules themselves might function as signal transduction molecules on T cells. For example, the cross-linking of MHC class I and II molecules on T cells causes increased calcium and increased cellular tyrosine phosphorylation (178, 179). Surprisingly, the cytoplasmic domain of the MHC class I molecule is not required for signal transduction (179). The physiological relevance of this form of activation is unknown, although it is possible that super-antigens, as ligands for MHC class II molecules, may be able to directly activate T cells that express MHC class II molecules. Further studies will be required to explore whether signal transduction by these molecules on T cells might contribute to certain forms of immunopathology.

The integrins VLA-4, VLA-5 and VLA-6 (very late antigen) are able to provide co-stimulatory effects to T cells (193–195). The ligands for these molecules are components of the extracellular matrix. The mechanism of the co-stimulatory effects are not yet clear but these interactions may be important in various aspects of tissue inflammation.

LYMPHOKINE-MEDIATED SIGNALING

While TCR stimulation prepares T cells for proliferation by promoting the movement of cells into the G1 phase of the cell cycle (201–203), completion of the cell cycle requires stimulation by growth-promoting lymphokines. Both IL-2 and IL-4 can induce proliferation in receptor-bearing cells. The receptor for IL-4 and the β chain of the IL-2 receptor belong to the cytokine receptor super-family (204–206). This family is quite large and includes the receptors for GM-CSF, IL-3, IL-5 and IL-6. These receptors share certain motifs in their extracellular domain, including the short stretch Trp-Ser-X-Trp-Ser (where X can be any amino acid) seen in all members of this family (207). None of these receptors encode enzymatic activity in their cytosolic tails, so it is believed that they couple indirectly to signal transduction pathways. Many members of this receptor family, including both the IL-2 and IL-4 receptor, activate tyrosine phosphorylation (208–210). While little is known about the early events following stimulation of cells with IL-4, a great deal of information is available regarding the molecular events underlying IL-2 effects.

Many of the same signal-transducing elements found to be activated by tyrosine kinase–growth factor receptors have been implicated in the action of IL-2. In particular, it has been shown that $p21^{ras}$ is activated, as measured by an increase in the ratio of the active GTP- to the inactive GDP-bound forms

(98, 211). In contrast, IL-4 stimulation does not activate *ras*, suggesting clear differences in the actions of these receptors in spite of their structural homologies (211). Phosphatidyl inositol 3-kinase (PI-3K) is activated rapidly in response to IL-2 stimulation (212). In fact, this enzyme also becomes associated with the IL-2 receptor in a ligand-dependent manner. PI-3K has been shown to associate with tyrosine kinase–growth factor receptors by a specific association between its SH2 domain and a tyrosine phosphate residue in the tyrosine kinase (81). However, the region of the IL-2 receptor responsible for PI-3K binding has not yet been defined. The serine/threonine kinase *raf-1* has also been shown to be activated in response to IL-2 (213).

A novel signal transduction pathway involving the generation of inositol phosphoglycan has also been implicated in the proliferative response to IL-2. This pathway was initially described as a component of the cellular response to insulin (214–216) and nerve growth factor (217). In responsive cells, the substrate is a distinctive glycosylphosphatidyl inositol (GPI) situated in the plasma membrane. On ligand binding, GPI is hydrolyzed by a specific PLC, generating the hydrophobic myristylated diacylglycerol (myr-DAG) and the soluble product inositol phosphoglycan (IP-glycan). In T and B lymphocyte lines expressing high-affinity IL-2 receptors, it has been found that IL-2 induces the hydrolysis of GPI in a dose-dependent manner, as measured by the generation of both products (218, 219). The induction is rapid, with peak effects seen at 2 minutes in the B lymphocyte line BCL_1 and at 30 seconds in the T lymphocyte line CTLL. The biological importance of GPI hydrolysis is suggested by two observations. First, GPI hydrolysis parallels proliferation in the BCL_1 line (218). In these cells, IL-4 antagonizes both the proliferative effects of IL-2 and the ability of IL-2 to induce GPI hydrolysis. Second, purified IP-glycan synergizes with IL-2 in CTLL cells, as demonstrated by a shift in the EC_{50} for IL-2 from 20 to 7 pM (219). This last study suggests that GPI hydrolysis is a rate-limiting component of the response to IL-2 but is not by itself sufficient for proliferation. The mechanism by which IL-2 induces GPI hydrolysis and the relationship (if any) with tyrosine phosphorylation is currently unknown.

Several lines of evidence suggest that tyrosine kinase activation is important in the action of IL-2. IL-2 induces rapid phosphorylation of a number of substrates in responsive cells (208, 210, 213), including the β chain of the IL-2 receptor itself (Table 5.1) (210, 220). A potential mechanism of coupling of the receptor to tyrosine phosphorylation is suggested by the finding that the β chain is physically associated with a tyrosine kinase activity. One component of this activity appears to be the cytosolic tyrosine kinase $p56^{lck}$, as shown by its co-precipitation with the receptor (221). In fact, $p56^{lck}$ is activated in response to IL-2 stimulation (222). The region of the β chain responsible for $p56^{lck}$ association is a serine-rich region in the cytosolic tail. Currently the significance of the association is unclear since deletion

mutants have shown that it is an acidic domain and not this serine-rich region that is required for signal transduction through the IL-2 receptor (223). Fung *et al.* have also explored the association of protein kinases with the high-affinity IL-2 receptor (224). They found both a tyrosine kinase and a serine/threonine kinase activity co-precipitating with the receptor. Interestingly, association with the tyrosine kinase required the presence of the same acidic region previously shown to be required for IL-2 signaling. In contrast, the serine/threonine kinase associated with a different region situated in the C-terminus which was also required for growth. The identity of these protein kinases has not yet been determined. These data indicate that the cytosolic tail of the β chain of the IL-2 receptor is composed of multiple domains which independently associate with a variety of protein kinases. The observation that domains involved in binding the protein kinases are also essential for growth suggests that these kinases are critical components of IL-2 signaling.

POTENTIAL ORPHAN PATHWAYS OF SIGNAL TRANSDUCTION IN T CELL ACTIVATION

Historically, the first well-characterized signal transduction pathway involved the cyclic nucleotide-dependent kinase family. However, today the functional role of cyclic nucleotides in lymphocyte activation and metabolism remains unknown. Cross-linking of the TCR with mAbs causes increases in cAMP concentration, and mitogenic lectins increase T cell cAMP levels (149, 225, 226). Lectins also increase the activity of cyclic nucleotide-dependent phosphodiesterases in T cells (227). The mechanisms and significance of these effects remain poorly understood.

A diverse group of phospholipases, in addition to PLC, have been shown to be activated in lymphocytes. Phospholipase D is activated in T cells after TCR stimulation (228). A magnesium-dependent PLC is activated in a subset of mouse B cells after antigen receptor cross-linking (229).

Various metabolic products of sphingomyelin may function in signal transduction. Ceramide is produced by sphingomyelinase and is an inhibitor of PKC. Ceramide analogs stimulate threonine phosphorylation of the EGF-R in A431 carcinoma cells (230). Ceramide treatment results in the differentiation of the HL-60 leukemic cell line (231). TNF-α stimulates a rapid increase in ceramide concentration in cell-free lysates of HL-60 cells, and an increase in ceramide-activated protein kinase activity (232, 233). Thus this pathway may be important for TNF signal transduction, and might play a role in T cell activation.

Diverse biochemical pathways are involved in lymphocyte activation. The emphasis of this chapter has been on the regulation of the signal transduction cascades that are thought to be most relevant to subsequent function of

T cells. It is likely that still other pathways of major significance are yet to be appreciated.

SIGNAL TRANSDUCTION AND APOPTOSIS

Apoptosis or programmed cell death is a multi-step biochemical process that eventuates in cell suicide (234). This process is distinct from necrosis—the death of cells from non-specific injury. Apoptosis is important in immune function, and is thought to be critical in T cell development as a means of selecting the T cell repertoire by removing self-reactive T cells. The bcl-2 proto-oncogene is expressed in the inner mitochondrial membrane, and the transforming mechanism of the bcl-2 oncogene appears to involve inhibiting cell death rather than by stimulating cell proliferation. Over-expression of the bcl-2 gene can protect thymocytes from many forms of cell death, such as corticosteroid, radiation and anti-CD3-induced apoptosis. However, bcl-2 cannot protect thymocytes from antigen-induced cell death, leading to the conclusion that there are multiple pathways in T cells leading to apoptosis (235).

The signal transduction pathways involved in the activation of programmed T cell death are only beginning to be understood. Antigen or anti-CD3 stimulation induces programmed cell death in thymocytes and transformed T cell lines (236–238). Thus, a basic paradox has emerged concerning how the signals delivered by the TCR result in either cellular proliferation or cell death. It is not yet resolved whether the differentiation state of the T cell determines the outcome, or whether there are differences in the signals delivered. Newell and co-workers found that mouse splenic T cells could be induced to proliferate or undergo cell death, depending on whether CD4 and the TCR were cross-linked independently or together, arguing that signals, in addition to the state of cellular differentiation, control the functional outcome (239). The signals delivered by cytokines also appear to select whether TCR stimulation by antigen leads to cell death or cell proliferation (240).

The induction of cell death in T cell hybridomas appears to be biphasic. The first phase is the establishment of cell cycle arrest at the G1/S interface, and occurs within an hour of anti-CD3 stimulation. This phase does not require extracellular calcium and is cyclosporin A insensitive. The second phase eventuates in cell lysis, and can be prevented by EGTA (ethyline bis[oxyethylenenitrilo]tetraacetic acid) and cyclosporin A, indicating that signal transduction involving calcium is required (241).

As was mentioned above, there appear to be distinct biochemical pathways leading to programmed T cell death. Both anti-CD3 and corticosteroids are able to induce cell death in thymocytes, yet these pathways are mutually antagonistic, as anti-CD3-induced cell death is prevented in the

presence of corticosteroids (242). Furthermore, cyclosporin A blocked anti-CD3-induced cell death but enhanced corticosteroid-induced cell death (242, 243). Agents that increase cAMP concentration cause apoptosis in thymocytes, while agents that activate PKC are able to inhibit programmed cell death (244, 245). The signals provided by IL-1 and anti-CD28 are also able to inhibit programmed cell death in some instances (246, 247). Thus, many receptor-mediated signal transduction events are able to inhibit TCR-induced cell death. Further studies will be required to determine whether these signals are involved in the decision to select T cell proliferation or cell death after antigen encounter. It is also possible that the inadvertent delivery of some of these signals could operate pathologically to prevent cell death, and thereby permit the emergence of autoreactive T cell clones and subsequent autoimmune disease.

Heat shock or ionizing radiation are able to induce apoptosis in lymphoma cell lines. Baxter and Lavin found that the induction of apoptosis in these lines was associated with dephosphorylation of a specific set of proteins (248). Okadaic acid, an inhibitor of protein serine/threonine phosphatases, was able to prevent apoptosis and the dephosphorylation of the proteins, suggesting that activation of phosphatases or loss of kinase activity are important in some forms of apoptosis.

The mouse accessory activation receptors Thy-1 and Ly-6 are able to trigger T cell proliferation and IL-2 production, as was mentioned above. This proliferative signal is dependent on the surface expression of the TCR. Nickas and co-workers found that Thy-1 and Ly-6 are also able to trigger programmed cell death. In contrast to T cell activation, the cell death induced by Thy-1 and Ly-6 occurs in the absence of the TCR, further indicating that the signals regulating cellular activation and apoptosis are distinct (249).

The Fas antigen is a 52 kDa transmembrane protein that has structural homology to tumor necrosis factor receptor and to the low-affinity nerve growth factor receptor. A ligand for this receptor has not yet been discovered. The antigen is expressed in various tissues, including thymocytes and a number of mature T cell lines. Cross-linking of the Fas antigen can lead to apoptosis in thymocytes and activated T cells (250). Recently, *lpr* mice were shown to have mutations in the Fas gene (251). As this strain of mice develops a lymphoproliferative disorder of T cells and is prone to autoimmune disease, the results indicate that the Fas antigen may have an important role in the form of programmed cell death that occurs in the thymus to remove autoreactive T cells. There is as yet no information concerning the signal transduction pathway controlled by the Fas receptor.

There is increasing evidence to indicate that HIV-1 infection may kill T cells by inducing apoptosis (252). CD4+T cells isolated from HIV-1-infected patients early in the course of the illness undergo apoptosis when stimulated

with antigen (247). Interestingly, the signal delivered by anti-CD28 was able to prevent cell death in these cells. Thus, the immunodeficiency associated with this infection may result, in part, from a signal delivered by HIV-1 to the T cell that programs the cells for suicide upon subsequent antigen activation. Together with the results of Newell and co-workers, who found that CD4 cross-linking with mAb could prime mature T cells to undergo cell death after anti-TCR stimulation (239), these findings suggest that one function of the CD4 receptor might be to modulate the TCR signal to result in cellular proliferation or cell death.

SIGNALS INVOLVED IN INDUCTION OF T CELL TOLERANCE

Tolerance is an example of a situation where engagement of the TCR, typically by self antigen, leads to inactivation of the receptor-bearing cells. In some cases self tolerance is maintained by the functional inactivation (anergy) of T cells expressing TCR specific for certain self antigens, while in other cases there is deletion of the reactive clones. Bretcher and Cohn first proposed a two-signal model of lymphocyte activation to explain how antigen-specific T cell activation could either result in T cell proliferation or anergy (253). Schwartz and colleagues have developed an *in vitro* cell culture model of anergy that displays many of the features predicted by the Bretcher and Cohn hypothesis. In this model, signal 1 is delivered by TCR occupancy, and signal 2 is delivered by the interaction of a co-stimulatory receptor on APC with a counter-receptor on T cells. If signal 1 is delivered in the absence of signal 2, a long-lasting state of unresponsiveness in mouse T_H1 clones and in some human CD4+ T cell clones is produced (13). The cells remain viable and will proliferate if exogenous IL-2 is added; however, the cells do not produce IL-2 or proliferate when subsequently exposed to APC pulsed with antigen. Conversely, T cell proliferation results when both signal 1 and signal 2 are delivered.

During the induction of anergy, signals are transduced by the TCR, including production of the second messengers calcium and Ins(1,4,5)P3, leading to the conclusion that signal 1 is normal during the induction of this form of anergy. Further, the TCR does not appear to be desensitized in anergic T cells, as antigen-unresponsive T cells transduce normal signals after re-exposure to antigen or after anti-CD3 mAb cross-linking. Efforts to date have not identified a specific co-stimulatory signal, although co-stimulatory signals do not appear to involve PLC (13). The transcription factors that are thought to regulate IL-2 gene expression may provide a biochemical signature to indicate the induction of unresponsiveness or T cell proliferation. When T cell clones are cultured in conditions that induce anergy, NFAT is not induced, and only one of two subunits of NK-κB are induced (254). These changes appear to be specific, as other transcription factors such as

AP-1 appear to be normally induced in the same cell cultures. The signal transduction cascades responsible for the induction of these transcription factors remain to be elucidated.

Several candidate receptors on APC have been proposed to function as the co-stimulatory receptor involved in T cell activation. In the mouse T_H1 model of anergy, the CD28 receptor appears to deliver a co-stimulatory signal, as anti-CD28 mAb is able to prevent the induction of non-responsiveness (140) Furthermore, a soluble fusion protein consisting of CTLA-4 and the IgG FcR can prevent or delay cardiac allograft rejection in the rat (255). Presumably, the mechanism responsible for this effect is that soluble CTLA-4 is able to prevent the interaction of B7 and endogeneous cell surface CD28, as CTLA-4 is a higher-affinity ligand for B7 than is CD28. The *in vivo* administration of mouse anti-CD2 mAb can result in the induction of unresponsiveness to antigens (168). In a rat allograft model of heart transplantation, the administration of anti-LFA-3 and anti-ICAM-1 mAbs is able to induce antigen-specific tolerance (184). Thus it is likely that multiple mechanisms may be employed to induce T cell anergy, and that several phenotypic states of unresponsiveness exist. To date, there is no evidence to indicate which mechanisms are operating *in vivo* in the various states of tolerance described above.

CONCLUSIONS

Substantial progress has been made in defining signal transduction pathways activated in response to stimulation through T lymphocyte cell surface receptors. Yet, in several important ways, gaps remain in our understanding of how T cells respond on the molecular level. Currently, many components have been identified in signal transduction through the various receptors. Further work will be required to order these components into coherent pathways and to determine in each case whether signaling proceeds in a linear manner from one component to the next or if multiple independent pathways operate in parallel. In addition, more experiments will be required to specify which of the signaling components are essential and which are dispensable. The goal of signal transduction studies is to derive a complete picture of how receptor engagement at the plasma membrane ultimately leads to the important nuclear events which underlie new gene expression. At the present, the mechanism by which the different signal transduction components interact has not been defined. However, the biochemical basis for interactions between different components is becoming better understood. Clearly, kinases—which regulate a multitude of substrates—and subdomains of proteins which participate in protein–protein interactions—like the SH2 domains—represent examples of how connections are made between different signaling molecules in responses mediated by many different receptors.

The picture which emerges from our current understanding of signal transduction appears quite complex. What is the utility to the immune system of having so many different elements involved in signaling? One likely explanation is that these signaling pathways originally evolved as part of the response to quite different stimuli. During evolution, receptors specific for T lymphocytes became coupled to several of these pre-existing signal transduction pathways and acquired their specificity based at least in part on the particular combination of pathways activated. Such a combinatorial mechanism of receptor specificity is likely since distinct receptors can activate some of the same signaling pathways yet retain their distinct effector function. The activation of $p21^{ras}$ by stimulation of both the TCR and the IL-2 receptor provides just one example. Another result of the complexity is to provide multiple levels at which signaling pathways can interact with each other. Such interactions may cause synergistic or inhibitory effects when two different receptors are activated simultaneously. Further studies of this form of receptor crosstalk may help to explain the complex interactions between the many different T lymphocyte receptors and accessory molecules. Such studies will undoubtedly help to extend our molecular understanding of the complex phenomena of T cell biology.

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